Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 149–153
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Insights into Vitamin D metabolism using cyp24 over-expression and knockout systems in conjunction with liquid chromatography/mass spectrometry (LC/MS)☆

Sonoko Masuda[a,*], Martin Kaufmann[a], Valarie Byford[a], Mian Gao[b], René St-Arnaud[c], Alice Arabian[c], Hugh L.J. Makin[d], Joyce C. Knutson[e], Stephen Strugnell[e], Glenville Jones[a]

[a] *Department of Biochemistry, Queen's University, Kingston, Ont., Canada*
[b] *Cytochroma Inc, Markham, Ont., Canada*
[c] *Shriner's Hospital, Montreal, Que., Canada*
[d] *Clinical Biochemistry, Bart's and the London, Queen Mary School of Medicine, London, UK*
[e] *Bone Care International, Madison, WI, USA*

Abstract

The development of novel gene expression systems for cytochrome P450s (CYPs) together with a revolution in analytical mass spectrometry with the emergence of liquid chromatography/mass spectrometry (LC/MS) has opened the door to answering some long-standing questions in Vitamin D metabolism. Our studies focused on: (1) elucidating the role of CYP24 in 25-OH-D_3 and 1α,25-$(OH)_2D_3$ metabolism; (2) exploring how DBP influences this process; (3) measuring 25-OH-D_3 metabolism in CYP24–knockout (CYP24–XO) cells and; (4) comparing 1α-OH-D_2 metabolism in the CYP24-XO mouse in vivo and in vitro. Methodology employed CYP24 over-expression and knockout systems in conjunction with state-of-the-art analytical LC/MS, diode array, and radioisotopic detection methods. We found that CYP24 metabolizes 25-OH-D_3 and 1α,25-$(OH)_2D_3$ at similar rates in vitro, but that for 25-OH-D_3 but not 1α,25-$(OH)_2D_3$, this rate is strongly influenced by the concentration of DBP. Unlike their wild type littermates, the administration of 25-OH-D_3 to CYP24-XO mice results in no measurable 24,25-$(OH)_2D_3$ production. When neonatal murine keratinocytes are prepared from wild type and CYP24-XO mice there was no measurable production of 24,25-$(OH)_2D_3$ or 1α,24,25-$(OH)_2D_3$ in CYP24-XO mice. Similar experiments using the same wild type and CYP24-XO animals and cells and [^{3}H]1α-OH-D_2 resulted in the apparent paradox that the Vitamin D prodrug was 25-hydroxylated in vivo but 24-hydroxylated in vitro.

Keywords: Cytochrome P450; CYP24; CYP24-knockout mouse; Vitamin D catabolism; DBP; LC/MS; 1α-Hydroxyvitamin D_2; 24-Hydroxylation

1. Introduction

The sequencing of the human genome has led to the realization that among a pool of sixty total cytochrome P450s (CYPs) [1], there are three known and possibly other uncharacterized CYPs dedicated to the metabolism of Vitamin D compounds. The cloning of these Vitamin D-related CYPs from a number of species has given enzymologists an unprecedented opportunity to study their catalytic properties in a variety of settings. We have chosen to generate over-expression and knockout systems to explore the properties of the CYP knowing the background activity of the host cell and also without the complexity of regulation [2]. This manuscript describes work with CYP24, where: (a) we have engineered a novel cell line which overexpresses human CYP24 in an environment (V79 Chinese hamster lung cell line) where there is no detectable baseline of CYP24 which can be used to study enzyme specificity and kinetics of the human CYP24 enzyme; and (b) in the CYP24-knockout (CYP24-XO) mouse [3] where we have studied animals in vivo after the administration of various [^{3}H] Vitamin D compounds: 1α,25-$(OH)_2D_3$, 25-OH-D_3 and 1α-OH-D_2; or (c) studied Vitamin D metabolism in keratinocytes prepared from neonatal CYP24-XO mice [4] and their heterozygous or wild type littermates.

☆ Presented at the Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July, 2003).

* Corresponding author. Tel.: +1-613-533-2498.
E-mail address: masudas@post.queensu.ca (S. Masuda).

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.03.094

In the current study, we have combined the use of several state-of-the-art techniques to investigate the metabolism of a wide variety of Vitamin D compounds. These techniques include in-line radio-scintillation detection, diode array spectrophotometry and newly established LC/MS protocols. LC/MS is an emerging technology which is being viewed as a powerful new tool combining excellent sensitivity with convenient sample preparation to facilitate the simultaneous analyses of multiple small molecules in a single run in a process known as 'metabolomics'. Thus far there are few examples of the application of LC/MS to Vitamin D metabolism and we combined it here with the cutting edge CYP expression systems to attempt to answer some important long-standing questions in the Vitamin D metabolic field. In particular, we wished to address the controversy around the exact physiological role of CYP24 in both 1α,25-$(OH)_2D_3$ and 25-OH-D_3 metabolism [5]; as well as explore the role of CYP24 in the metabolism of analogs such as 1α-OH-D_2 which is both 24- and 25-hydroxylated in vivo [6].

2. Methods and materials

2.1. Cell culture and incubation

HPK1A-*ras*, V79-CYP24, and V79-4 cells were maintained in 150 mm cell culture plates with Dulbecco's Modified Eagle Medium (DMEM) [Gibco] supplemented with 10% (v/v) FBS [ICN] and 1% (v/v) antibiotic/antimycotic [Gibco]. Primary keratinocytes harvested from CYP24-XO, heterozygous and wildtype mice were maintained in 60 mm cell culture plates. At 80–90% confluence, cells were subcultured into six well plates. After recovery, the cells (except V79-CYP24) were induced with 10 nM 1α,25-$(OH)_2D_3$ for 18 h and incubated in DMEM supplemented with 1% (w/v) bovine serum albumin (BSA) [Boehringer Mannheim] and 1% (v/v) antibiotic/antimycotic containing variable amounts of 1α,25-$(OH)_2D_3$/25-$(OH)_2D_3$ or [1β-^{3}H]1α,25-$(OH)_2D_3$/[26,27-^{3}H]25-OH-D_3 (1, 100, 500, 750 nM, 1, 5, 7.5 and 10 μM), in triplicate assays for 24 h (See Fig. 1). Incubations were also carried out at fixed concentrations (1 or 10 μM) over a 48 h time course (0, 4, 8, 12, 24 and 48 h) with both substrates. The 2 μl well volume was supplemented with 2 ml of 100 mM 1,2-dianilinoethane antioxidant [Sigma]. No-cell, dead-cell, and untransfected V79-4 cell controls were incubated with the 10 or 1 μM substrate stock solution. After the incubation, 1α-OH-D_3 (or Vitamin D_3 for incubations with 25-OH-D_3) was added as an internal standard to assess recovery from extraction. In selected incubations, DBP was added to the incubation medium at concentrations of 0.1–0.5 μM (1–5% physiological).

2.2. Extraction and HPLC

Media and cells from cell culture or tissues from mice were extracted using a modification of the Bligh and Dyer extraction. Normal phase HPLC was carried out on an Alliance 2695 separations module with 996 photodiode array detector [Waters]. Where needed, radioactivity analysis was carried out in-line. Separation was facilitated by an isocratic solvent system of 91/7/2 or 96/3/1 hexane/isopropanol/methanol at 1 ml/min on Zorbax-Sil columns [Agilent]. The Bligh and Dyer aqueous phase was subjected to 0.015% (v/v) glacial acetic acid (GAA) and re-extracted with 5 ml methylene chloride after acidification by the addition of 0.015% (v/v) glacial acetic acid (GAA). The aqueous layer re-extracts were analyzed using a Zorbax-SB C18 column [Agilent] with an

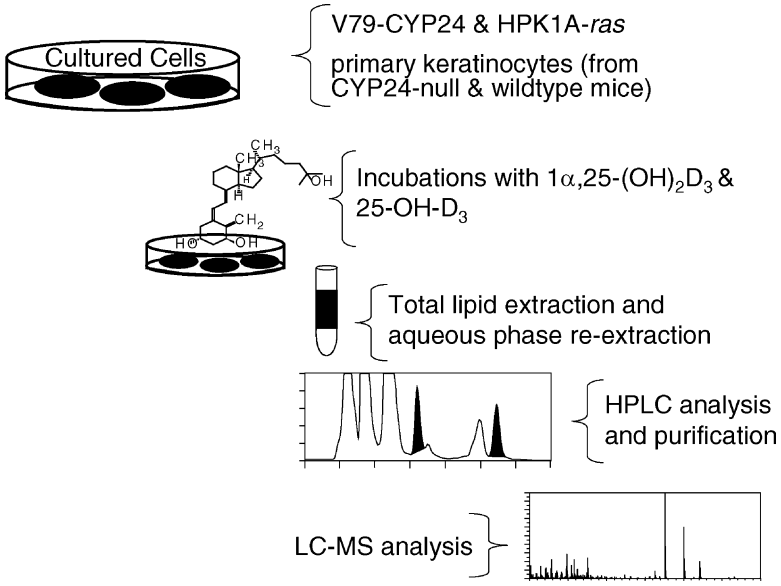

Fig. 1. LC/MS-based methodology for the study of Vitamin D metabolism.

acetonitrile/water/GAA-based gradient system at 1.0 ml/min. Catabolites were identified on the basis of the characteristic Vitamin D *cis*-triene chromophore (λ_{max} = 265; ε = 18,300) and co-chromatography with authentic synthesized standards for 1α,25-$(OH)_2D_3$, 25-OH-D_3, 1α,24,25-$(OH)_3D_3$, tetranor-1α,23-$(OH)_2D_3$, calcitroic acid and 24,25-$(OH)_2D_3$. Catabolite identity was confirmed by LC/MS while quantification was based upon diode array detection or LC/MS.

2.3. LC/MS

Peaks possessing the Vitamin D chromophore were collected from the above chromatographic analyses. The fractions were subjected to a methanol/water/GAA-based gradient system (Zorbax-SB C18 [Agilent], 0.2 ml/min) for injection into a Quattro Ultima electrospray mass spectrometer [Micromass] to confirm the identity of the lipid- and water-soluble catabolites.

3. Results

Initial experiments involved establishing methods for the LC/MS detection of all known C-24 oxidation metabolites of 1α,25-$(OH)_2D_3$ and 25-OH-D_3 [7]. Fig. 2 illustrates the LC/MS mass spectra of 25-OH-D_3 and its five C-24 oxidation metabolites. Detection limits for all metabolites were in the picogram range permitting metabolism studies on the CYP24 expressing cells easily down to a substrate concentration of 100 nM.

Enzymatic properties of a V79 cell line stably transfected with human CYP24 were compared with the human keratinocyte, HPK1A-ras, a naturally occurring CYP24 over-expressing cell line. Both cell lines revealed very similar data when 1α,25-$(OH)_2D_3$ or 25-OH-D_3 were used as substrate. Fig. 3 shows the data when HPK1A-ras cells are incubated with 25-OH-D_3 or 1α,25-$(OH)_2D_3$ at a substrate concentration of 10 μM for 24 h. Both cell systems generated all expected intermediates from 1α,24,25-$(OH)_3D_3$ to calcitroic acid and 25-OH-D_3 to a truncated C_{23} acid (shown in Fig. 2) over the full concentration range 100 nM to 10 μM used. At lower substrate concentrations both compounds were efficiently converted to terminal catabolites. Somewhat surprisingly, 1α,25-$(OH)_2D_3$ and 25-OH-D_3 were catabolized at similar rates when an artificial culture medium devoid of fetal calf serum but containing 1% BSA was used. On the other hand when human recombinant DBP was added to the external medium at concentrations ranging from 1 to 5% of physiological there was a significant reduction in the rate of 25-OH-D_3 metabolism but not of 1α,25-$(OH)_2D_3$ metabolism (data not shown). The effect is consistent with sequestration of 25-OH-D_3 but not 1α,25-$(OH)_2D_3$ by DBP on the exterior of the CYP24-expressing cell.

Another way to study the actions of CYP24 in the natural setting is to delete its gene. Using CYP24-XO mice generated by St-Arnaud's group in Montreal [3], we previously showed that these mice, as compared to

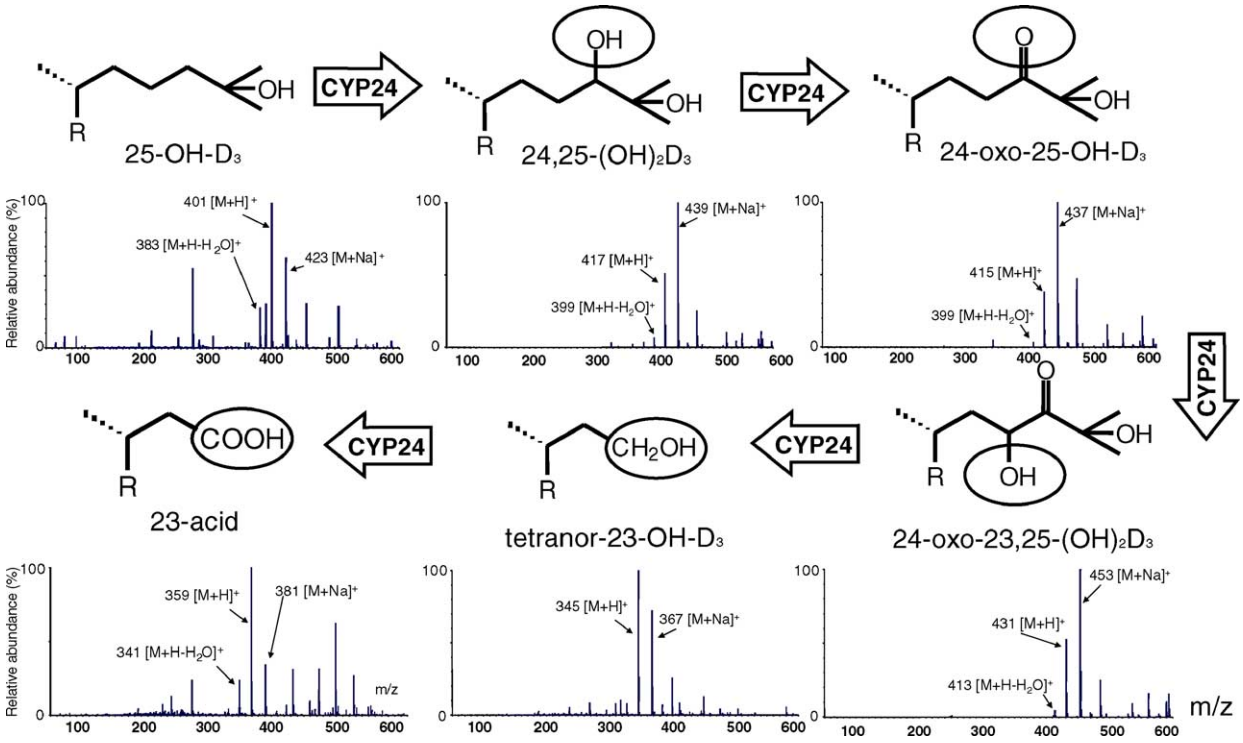

Fig. 2. LC/MS analysis of the metabolites of 25-OH-D_3.

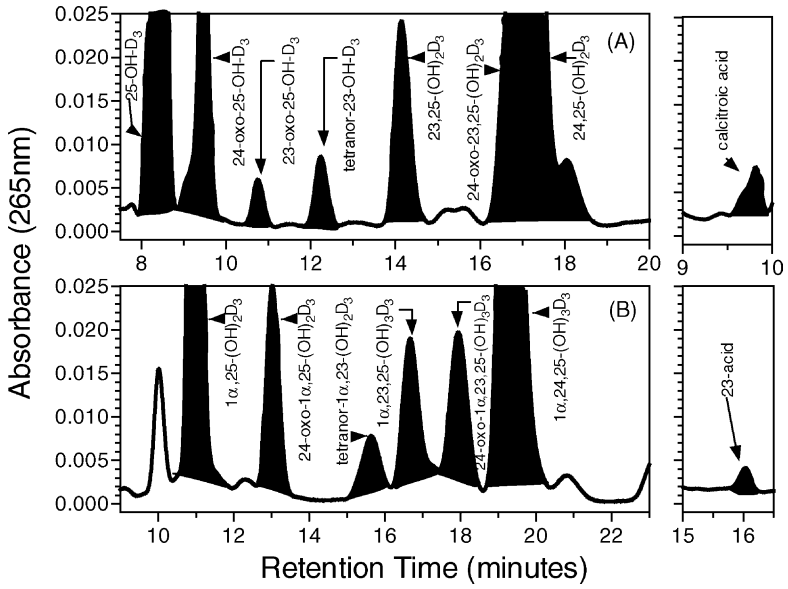

Fig. 3. HPLC analysis showing the metabolism of (A) 10 μM 25-OH-D_3 and (B) 1α,25-$(OH)_2D_3$ by HPK1A-ras cells in the absence of FBS (source of DBP) but in the presence of BSA.

heterozygous and wild type littermates, have a reduced capacity to clear a bolus dose of [1β-^{3}H]1α,25-$(OH)_2D_3$ from the bloodstream and tissues [8]. In these studies, we administered [26,27-^{3}H]25-OH-D_3 to CYP24-XO mice and followed the production of 24-oxidation products including 24,25-$(OH)_2D_3$ by LC of blood and other tissues. We observed a total absence of 24,25-$(OH)_2D_3$ in CYP24-XO mice whereas heterozygous mice showed a significant peak of the metabolite in all tissues tested. Fig. 4A shows analysis of the kidney tissue from CYP24-XO and control animals. When primary neonatal keratinocytes were prepared from CYP24-XO and +/− animals, the findings were essentially the same with 24,25-$(OH)_2D_3$ only formed when the CYP24 gene was expressed. Interestingly, despite being cultured under low Ca conditions the CYP24-XO keratinocytes failed to show significant 1α,25-$(OH)_2D_3$ production even though the masking action of CYP24 was absent.

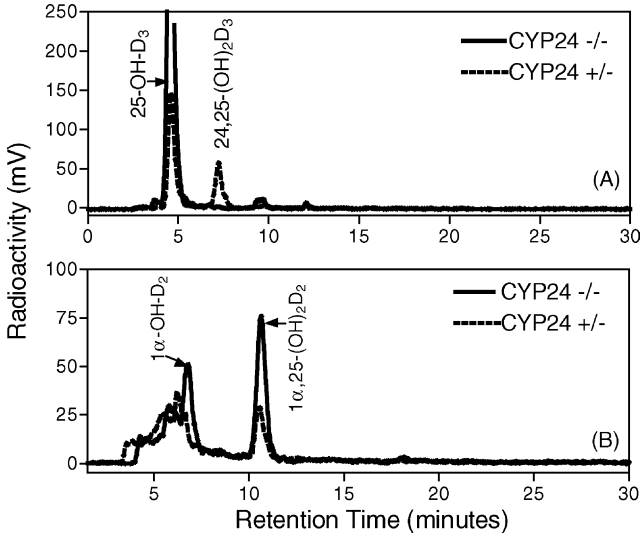

Fig. 4. HPLC analysis of kidney tissue from CYP24-XO mice or their heterozygous littermates after administration of either (A) 5 μCi of 25-OH-D_3 or (B) 5 μCi of 1α-OH-D_2.

We also explored the use of the CYP24-XO mouse in the study of the metabolism of Vitamin D analogs. The analog we chose to study was the BCI analog, 1α-OH-D_2 which is both 24- and 25-hydroxylated in vivo and which has been extensively studied in target cells in culture [6]. Using [9,11-^{3}H]1α-OH-D_2 at high specific activity (40 Ci/mmol), we administered a single 5 μCi dose of analog to CYP24 −/−, +/− and +/+ animals. At various time-points and in all tissues tested, [9,11-^{3}H]1α-OH-D_2 was converted to [9,11-^{3}H]1α,25-$(OH)_2D_2$ but no [9,11-^{3}H]1α,24-$(OH)_2D_2$ was evident. Fig. 4B shows representative results from analysis of the kidneys of the two groups of animals. In reviewing the data, there is considerable animal-to-animal variability but there is a clear trend towards higher [9,11-^{3}H]1α,25-$(OH)_2D_2$ levels in CYP24-XO animals. This may be a consequence of the lack of CYP24 to catabolize the putative active form. In this regard, it should be noted that primary neonatal keratinocytes from these same animals show a CYP24-dependent ability to synthesize 1α,24-$(OH)_2D_2$ and other side-chain hydroxylated metabolites from 1α-OH-D_2 as well as a spectrum of catabolites. From the data it is presumed that in vitro 24-hydroxylation of 1α-OH-D_2 and the catabolism of all side chain hydroxylated products is due to CYP24.

4. Discussion

The development of LC/MS-based methodology to detect Vitamin D metabolites combined with the latest recombinant

cytochrome P450 protein expression systems provides powerful tools to look at problems in Vitamin D metabolism. In the work described here, we used these new models and technology to investigate the properties of the cytochrome P450, CYP24 in a variety of settings: in vivo, naturally occurring in cells in vitro and transfected into host cells not expressing native CYP24 in vitro. The results all reinforce what we already know about CYP24 namely that it carries out the 5-step C24-oxidation pathway which converts 1α,25-$(OH)_2D_3$ or 25-OH-D_3 into side chain truncated acids. The finding of the C_{23}-acid from 25-OH-D_3 is the first report of this metabolite and is made possible by the sensitivity and convenience of LC/MS.

The finding of a similar rate of metabolism of 1α,25-$(OH)_2D_3$ or 25-OH-D_3 by CYP24 is novel, though there are hints in the literature from *Escherichia coli* expression systems that this might be the case [9]. In fact, Taniguchi et al. [9] concluded that 25-OH-D_3 is the preferred substrate for CYP24, though there are claims from other workers that Taniguchi et al's methodology for 1α,25-$(OH)_2D_3$ is greatly flawed and measurements for rate of 1α,25-$(OH)_2D_3$ catabolism are underestimated [5]. The work described here provides documentation that DBP present in the culture medium has a profound influence on the rate of 25-OH-D_3 metabolism but not 1α,25-$(OH)_2D_3$ metabolism presumably sequestering the 25-OH-D_3 but not 1α,25-$(OH)_2D_3$ on the exterior of the cell. The work also reinforces a conclusion that reconstituted cell-free purified protein preparations of CYP24 in the test-tube cannot simulate the true physiological situation and lead to misleading conclusions. Indeed, the concepts developed here using CYP24 expressed inside a non-renal cell do not provide insights on how 25-OH-D_3 is able to traverse the plasma membrane and get 1α- and 24-hydroxylated to give the metabolites 1α,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. However, the work with these cells provides good insight into why 1α,25-$(OH)_2D_3$ is so readily catabolized inside target cells without the influence of DBP.

The availability of the CYP24-XO mouse opens up new approaches to determine the involvement of CYP24 in Vitamin D analog metabolism. The model is particularly useful to discern nuances of the metabolism of the marketed prodrug analog 1α-OH-D_2 [6]. It is widely believed that 1α-OH-D_2 is 25-hydroxylated in the liver to give 1α,25-$(OH)_2D_2$ but evidence acquired in several species suggests the additional formation of an alternative bioactive product 1α,24S-$(OH)_2D_2$. As expected, the in vivo data acquired here suggest CYP24 plays no role in 1α,25-$(OH)_2D_2$ production because there is no lack of the metabolite in the blood or tissues. However, the data also show that CYP24 may play a role in 1α,25-$(OH)_2D_2$ catabolism, and if anything 1α,25-$(OH)_2D_2$ levels are elevated in CYP24-XO animals. The data are unhelpful on the origins of the 1α,24S-$(OH)_2D_2$ since there was no detectable production of 1α,24S-$(OH)_2D_2$ in wild type, heterozygous or knockout mice. It is assumed that the production of 1α,24S-$(OH)_2D_2$ is achieved only at analog concentrations not reached here or that there are species differences in cytochrome P450 complements which play a role here. Another aspect of this story is that CYP24-containing tissues such as primary neonatal keratinocytes do generate 1α,24S-$(OH)_2D_2$ and its further catabolites from 1α-OH-D_2 in vitro. The production is abolished in CYP24-XO keratinocytes leaving one to conclude that CYP24 must be involved in both the step of activation to 1α,24S-$(OH)_2D_2$ and steps of its catabolism to polyhydroxylated inactive products [4,10]. The new knowledge will be useful to rationalize the mechanism of 1α-OH-D_2 prodrug activation and to the continued development of synthetic 1α,24S-$(OH)_2D_2$ as a drug. In the process these studies show the validity of using customized CYP24-deficient mice in Vitamin D analog development.

References

[1] Cytochrome P450, Homepage at http://drnelson.utmem.edu/humP450.aln.html.

[2] S. Masuda, M. Gao, A. Zhang, M. Kaufmann, G. Jones, Importance of cytochrome P450-mediated metabolism in the mechanism of action of vitamin D analogs, in: J. Reichrath, M. Friedrich, W. Tilgen, (Eds.), Recent Results in Cancer Research: Vitamin D analogs in Cancer Prevention and Therapy, vol. 164, Springer-Verlag, Germany, 2003, pp. 189–202.

[3] R. St-Arnaud, A. Arabian, R. Travers, F. Barletta, M. Raval-Pandya, K. Chapin, J. Depovere, C. Mathieu, S. Christakos, M.B. Demay, F.H. Glorieux, Deficient mineralization of intramembranous bone in vitamin D-24-hydroxylase-ablated mice is due to elevated 1,25-dihydroxyvitamin D and not to the absence of 24,25-dihydroxyvitamin D, Endocrinology 141 (2000) 2658–2666.

[4] S. Masuda, A. Arabian, J. McCaig, M. Kaufmann, S.A. Strugnell, J.C. Knutson, R. St-Arnaud, G. Jones, CYP24-null keratinocytes demonstrate that CYP24 is responsible for activation and inactivation of 1α(OH)D_2, J. Bone Miner. Res. 17 (2002) S396.

[5] G. Jones, H.S. Tenenhouse, 1,25-$(OH)_2D_3$, the preferred substrate for CYP24, J. Bone Miner. Res. 17 (2001) 179–181.

[6] S. Strugnell, V. Byford, H.L.J. Makin, R.M. Moriarty, R. Gilardi, L.W. LeVan, J.C. Knutson, C.W. Bishop, G. Jones, 1α,24(S)-dihydroxyvitamin D_2: A biologically active product of 1α-hydroxyvitamin D_2 made in the human hepatoma, Hep3B, Biochem. J. 310 (1995) 233–241.

[7] G. Makin, D. Lohnes, V. Byford, R. Ray, G. Jones, Target cell metabolism of 1,25-dihydroxyvitamin D_3 to calcitroic acid: Evidence for a pathway in kidney and bone involving 24-oxidation, Biochem. J. 262 (1989) 173–180.

[8] G. Jones, V. Byford, A. Arabian, R. St-Arnaud, Altered pharmacokinetics of 1α,25-$(OH)_2D_3$ in blood and tissues of the CYP24-null mouse, J. Bone Miner. Res. 15 (2000) S199.

[9] T. Taniguchi, T.A. Eto, H. Shiotsuki, H. Sueta, S. Higashi, T. Iwamura, K.I. Okuda, T. Setoguchi, Newly established assay method for 25-hydroxyvitamin D_3 24-hydroxylase revealed much lower Km for 25-hydroxyvitamin D_3 than for 1α,25-dihydroxyvitamin D_3, J. Bone Miner. Res. 16 (2001) 57–62.

[10] R.L. Horst, J.A. Omdahl, S. Reddy, Rat cytochrome P450C24 (CYP24) does not metabolize 1,25-dihydroxyvitamin D_2 to calcitroic acid, J. Cell Biochem. 88 (2003) 282–285.